THE TOXICITY OF PHYSALIA NEMATOCYSTS[1]

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The siphonophore *Physalia physalis* (Portuguese-man-of-war) possesses a well-merited evil reputation throughout its geographical range. Contact with the tentacles of this animal is always painful to man and may result in vasomotor dysfunction and collapse. Although toxic substances have been previously isolated from *Physalia* tentacles (Richet and Portier, 1936), there appears to have been no examination of the toxic compounds which originate specifically within the nematocyst.

Phillips (1956) described a modification of the method of Glaser and Sparrow (1909) by which the nematocysts of *Metridium* could be isolated, washed and discharged into distilled water. The methods presented here are similar to his. It is our object to present details of the separation of nematocysts from *Physalia* and preliminary data on the composition and toxicity of the separated components. Although *Physalia* is a colonial form, for simplicity, members of the colony will be referred to as if they were anatomical parts and the whole colony as a single entity.

Materials

Physalia appears on southeast Florida beaches during periods of prolonged on-shore winds of greater than usual intensity. Locally these winds may be expected seasonally, from October through March. Small animals generally appear early in the season. Specimens of *Uca pugilator* were purchased from a commercial distributor in the vicinity of Englewood, Florida.

Methods

Specimens were collected as they stranded and were placed in clean sea water to remove sand. The fishing tentacles were removed, combined with the tentacles from other animals, and allowed to autolyze at 4° C. for 24 to 48 hours. Then the mixture was diluted with one or more volumes of sea water and put through graded screens of 24 and 115 meshes per inch. This removed most of the muscle and connective tissue of the tentacle and permitted passage of undischarged nematocysts. The screened suspension was allowed to settle overnight in the cold. The supernatant solution was decanted and discarded. The residue, which was composed chiefly of nematocysts, was centrifuged at 300–400 g for 15 to 30 minutes. The supernatant solution was again discarded and the residue re-suspended in sea water. These processes were continued until injection of 0.1 ml. of the supernatant solution into the hemocoele of the fiddler crab, *Uca*, was without apparent

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effect. The nematocyst suspension at this time (Fig. 1) was almost completely free of tentacular tissue fragments and contained approximately 55 million nematocysts per wet gram, very few of them discharged. Nematocysts ranged in size from 8.8 to 42.3 micra. They fell into two size groups: one with a mean diameter of 11.3 micra made up 23% of the total sample. The remainder varied about a mean diameter of 26.8 micra. The packed nematocysts were frozen and stored at − 5° C. Nematocysts were still reactive after 20 weeks of frozen storage. An initial sample of 3.4 liters of isolated fishing tentacles yielded 60 grams of packed wet "purified" nematocysts.

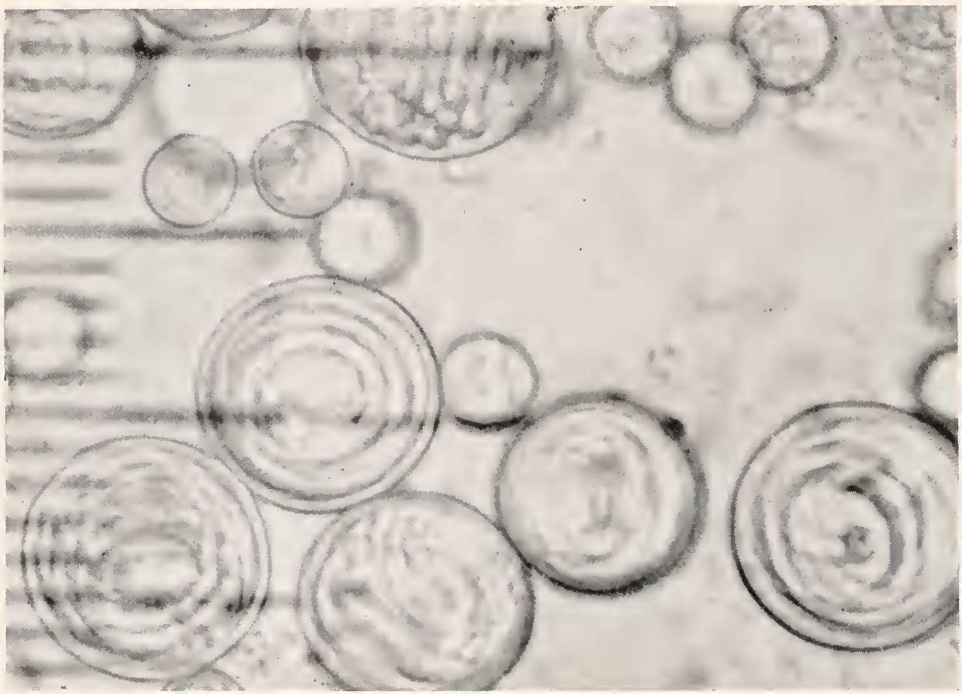

FIGURE 1. Photomicrograph of isolated, purified, still-reactive nematocysts of *Physalia*. × 1400.

The contents of the isolated nematocysts were liberated by homogenization in a chilled Potter-Elvejhem homogenizer. Amphibian Ringer's, sea water or distilled water may be used as the diluent. The sample was examined microscopically at intervals and homogenization was continued until about 90% of the capsules were fragmented (Fig. 2). The homogenate was centrifuged at 600 g for ten minutes to separate capsules and capsular fragments from the diluted capsular contents. The supernatant solution was cloudy, yellowish-white in color, and extremely toxic to crabs, fish and small mammals. Precautions must be taken to avoid exposure of skin to contamination by any mixture, wet or dry, which contains undischarged nematocysts. Nematocysts on laboratory surfaces or clothing retain their reactivity for at least two weeks as unpleasant reminders of previous careless-

ness. Nematocysts on the tentacles of large living *Physalia* may occasionally penetrate heavy-gauge surgical gloves. Surfaces, clothing and skin can be decontaminated by the application of 95% ethanol. Although this treatment does not reduce the pain of stings already received, it appears to prevent the discharge of additional nematocysts.

Fiddler crabs (*Uca pugilator*) have been employed for initial screening of toxic extracts. Doses of 0.1 ml. of material to be assayed were injected into the hemocoele through the articular membrane of the third walking leg. When sea

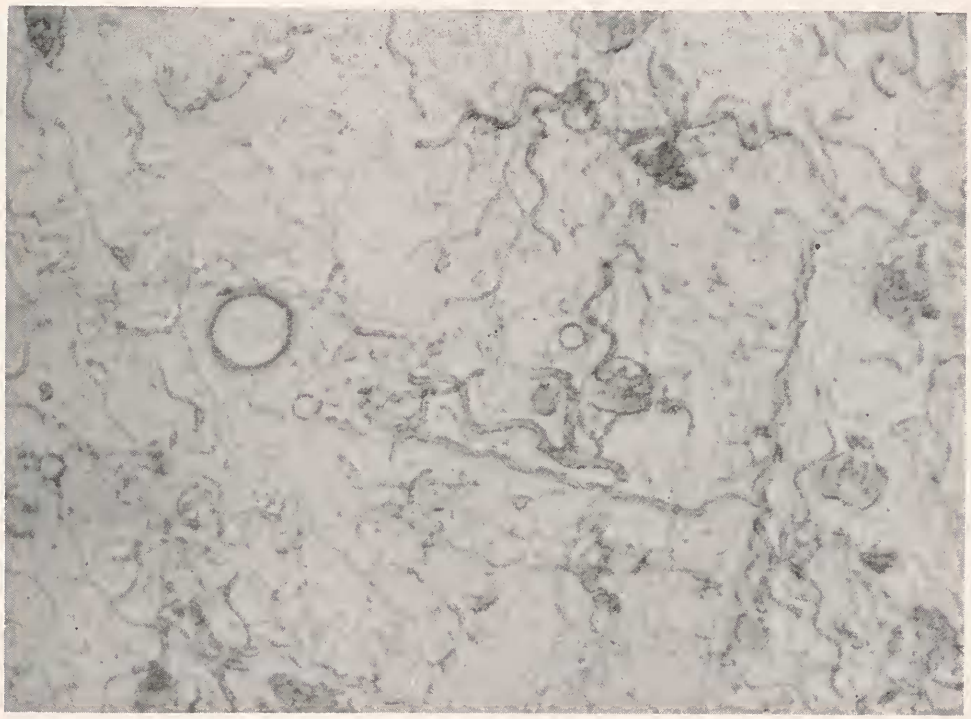

FIGURE 2. Photomicrograph of homogenized nematocysts, × 250. Fragments of capsules, everted tubules and tubule fragments constitute most of the visible formed elements. At least 90% of the capsules have been discharged.

water alone was administered by this route no effect was produced, but when capsular contents were present in the sea water, paralysis and death ensued. Ten animals were routinely injected with each extract to be assayed. Other animals employed in toxicity determination and evaluation include several species of fish, the frog, and the heart of the clam *Mercenaria campechiensis* (frequently designated *Venus mercenaria*). Acute toxicity studies were done on 30-gram, male Swiss mice, according to the method of Deichmann and LeBlanc (1943).

Total nitrogen was determined by the micro-Kjeldahl method; moisture by co-distillation with toluene; and ash by incineration. Dry samples of known weight were hydrolyzed in 6 N HCl in a sealed capsule at 100° C. for 24 to 36 hours. The

amino acid content of the neutralized hydrolysate was determined by two-dimensional chromatography on Whatman No. 1 paper. Two-dimensional chromatograms employed n-butanol, acetic acid and water (4:1:5) as the first solvent and water-saturated phenol as the second solvent. Chromatograms were developed in 0.2% ninhydrin in acetone. The approximate concentrations of amino acids in various samples were estimated by size and density of the amino acid spots on the finished chromatograms.

Results

The distribution of amino acids in *Physalia*, and their approximate concentrations are shown in Table II. The predominant amino acids in undischarged

Table I

Composition of entire colonies and of component parts of Physalia

Material	Moisture	Fat	Solids	Ash	% Total N
Entire, living *Physalia*	82.2%	0.23%	17.5%		
Floats, only	88.5		11.48		10.94%*
Fishing tentacles	88.07		11.93		
Gonozooids	88.78		11.22		
Undischarged nematocysts**	77.8		22.2	3.35	2.58
Capsule contents	97.54		1.46		0.598
Discharged capsules (residue)	88.0		12.0		1.48
First wash of discharged capsules					0.12

* This determination refers to dry material; all other N determinations are wet-weight basis.
** This was a standard preparation numbering 55.2 millions of capsules per milliliter.

Table II

Amino acids of Physalia

Amino acid*	Entire Physalia	Fishing tentacle	Undischarged nematocysts	Nematocyst contents	Discharged capsules
Cystine	X	X	X	X	X
Cysteine	X	X	X	X	X
Glutamic acid	XXX	XXX	XXX	XXXX	X
Glycine	X	XX	XX	X	XX
Alanine	X	X	XX	X	XXX
Tyrosine	0	0	0	0	0
Proline	X	X	XX	0	XXX
Hydroxyproline	X	X	X	X	X
Leucine	X	X	X	X	X
Isoleucine	X	X	X	X	X
Methionine	X	X	X	0	X
Lysine	X	X	X	X	0
Threonine	X	0	0	0	0
Aspartic acid	X	X	0	0	0
Histidine	X	XX	0	0	0
Serine	X	X	0	0	0

* X means not more than 2.5 micrograms of amino acid in 50 micrograms dry hydrolysate. 0 = no spot.

nematocyst capsules appear to be glutamic acid, glycine, alanine and proline. Of these, glutamic acid is chiefly a constituent of the fluid contents of the capsule, and the others occur chiefly in the solid components of the capsule wall. Lysine, present in the nematocyst complex in small quantity, apparently also is concerned only with the fluid contents. Aspartic acid, histidine, threonine and serine are present in the intact animal, but are apparently not present in the capsular complex.

When an active extract in sea water was administered to the fiddler crab, the response was immediate and predictable. When returned to the container, the injected crab made a short, abrupt run, stopped precipitately, contracted the extensors of the walking legs vigorously. This made the animal appear to rise on tiptoes. It remained motionless during the imperceptible relaxation which culminated in death. If the crabs were handled after relaxation began, responses were limited to the eyestalks and to very slow movements of the walking legs. The animals appeared to be paralyzed. If legs of "paralyzed" crabs were crushed with a hemostat, the number of legs autotomized was only one-third that observed in uninjected crabs similarly treated.

Activity of the fluid contents of the capsule was markedly decreased by heating to 60° C. for 15 minutes, by precipitation with acetone or by extraction with ether. The toxin was non-dialyzable. It was positive to ninhydrin and negative to Benedict's reagent, both before and after acid hydrolysis. Activity persisted without significant quantitative change for at least two months when stored at − 5° C. When the capsule contents were precipitated by alcohol and then assayed on crabs, a qualitative fractionation of the total activity was observed. Before treatment with alcohol the extract produced immediate death of test crabs throughout the effective concentration range. After precipitation in alcohol and re-solution of the precipitate in sea water, or variation of the pH, the lethal response was delayed as much as 24 hours, but the extract produced immediate autotomy of the walking legs. Similarly, adsorption of the toxin on paper and subsequent elution released only the autotomy-producing activity. The residue on the paper, as well as the eluate, remained ninhydrin-positive.

The approximate lethal dose for mice of a toxin sample which contained 0.201% total N was 2.1 ml./kilo. when the material was injected subcutaneously (12 mice) and 0.037 ml./kilo. when it was injected intraperitoneally (23 mice). Subcutaneous injection caused depression after about two hours, and death, apparently due to respiratory failure, occurred 12 to 18 hours after injection. Post-mortem examination of a single mouse immediately after it had stopped breathing showed the heart to be still beating, indicating that death was due primarily to respiratory failure. After intraperitoneal injection there was an immediate onset of intoxication—reminiscent of the almost instantaneous response of the fiddler crab. Symptoms included increased activity and tremors probably due to local irritation. After 10 minutes there were ataxia, decreased muscle tone, flaccid paralysis, slowed and labored breathing, defecation, aphrodisia, marked myosis, cyanosis, anoxic convulsions and death. Survival time was 1 to 48 hours, depending on the dose administered. Post-mortem examination showed the following gross pathology: lungs, blanched; heart, contracted, especially the left ventricle; hemorrhagic edema in the peritoneal cavity; skin of nose and ears very white; cornea, cloudy; colon, no formed stools; urinary bladder, empty.

A dose of 0.5 ml. of crude toxin containing 2.43 μ gm. N per ml. was uniformly lethal when injected into the left ventral lymph sac of each of eight frogs (*Rana pipiens*). Within five minutes the white ventral surface of the frog developed irregular red patches which suggested a localized hemodynamic response if not actual escape of blood cells from the capillaries. Breathing became rapid and shallow. Righting and postural reflexes deteriorated progressively during the first hour. At the time the animal first failed to respond to visual stimulation (about 30 minutes), he could be turned over if stimulation of peripheral end organs were minimized. At this time, spinal reflexes appeared to be normal. After 75 minutes, breathing movements ceased and spinal reflexes disappeared. Electrical stimulation of the sciatic nerve elicited no response at this time, but direct stimulation of the gastrocnemius muscle showed it to be normally reactive. The heart continued to beat for 12 to 24 hours. Large amounts of lymph accumulated subcutaneously in the abdominal area. Viscera were hyperaemic, bladder and intestine, empty and in several instances large amounts of bloody intraperitoneal fluid were observed.

Fish responded immediately to intramuscular injection of lethal doses of crude toxin by hyperventilation and rapid swimming. Petechiae often appeared at the sclero-corneal junction. After five minutes to several hours, depending on dosage, the fish became disoriented, sank to the bottom of the tank, and died after a period of one to four hours. This response is typical of the pilchard *Harengula humeralis*, silversides *Hepsitia stipes* Müller and Troschel, and *Fundulus heteroclitus*. These species exhibited chromatophoric responses to injection, usually blanching at the immediate site of the injection and darkening over the general body surface. Examination of two pilchard which had fallen to the bottom of the tank immobilized, showed the heart of each to be beating normally. No abnormal effects were observed after intraperitoneal injection of a lethal dose of toxin. Washed erythrocytes of the mullet (*Mugil cephalus*) did not hemolyze when incubated at 37° C. with several dilutions of crude toxin.

The heart of the clam *Mercenaria campechiensis*, isolated by the method of Welsh and Taub (1948) responded to administration of crude toxin at a concentration of 5.2 μ gm. N/ml. of bath. The pattern of response was similar to that obtained with acetylcholine, *i.e.*, cessation of beat in diastole. The crude toxin appeared to produce irreversible changes which prevented the heart from giving an equivalent response to a similar dosage later. Administration of the crude toxin did not modify the response of the heart to acetylcholine.

Discussion

The crude toxin of the nematocyst is apparently a protein complex or is associated with a protein. The lethal components obscure secondary or side reactions. After warming to 60° C., adsorption on paper and subsequent elution, precipitation with ethanol and subsequent re-solution, manipulation of pH or other mild treatments, at least two fractions of the total activity were resolved and then exerted separate effects on test animals. Welsh (1956) has shown that many substances, including extracts of *Physalia* and various other coelenterates, modify the autotomy reflex in crustaceans. It is therefore of considerable interest that a compound

which caused autotomy appeared in the capsular contents only after this material had been subjected to drying, heating, or other procedures which cause denaturation. This effect has not been produced by unmodified extracts.

Lenhoff, Kline and Hurley (1957) have described a characteristic chemical composition of nematocyst capsules of other coelenterates. They have suggested, together with Phillips (1956), that the capsule is similar in chemical composition to the collagenous group of proteins of higher animals. In homogenized preparations of *Physalia* nematocysts, the capsules tend to retain their general shape though they be ruptured or even broken completely in two. This observation provides a certain amount of support for the concept that the capsule wall is semi-rigid.

Our data suggest that *Physalia* differs from *Metridium* in that the amino acid spectrum of the capsule contents differs both qualitatively and quantitatively from that of the capsule wall. Apparently no hexose constituents are present in *Physalia* although Phillips describes hexoseamines from *Metridium*.

Our methods of isolation of nematocysts of *Physalia* require no other diluent than sea water, with which the nematocysts are presumably normally in contact. This avoids the introduction of extraneous salts and may contribute to the long persistence of reactivity we have observed. We have elected to liberate the capsule contents by homogenization rather than to await the considerable time that may be required for normal discharge. The lability of *Physalia* toxin necessitates a minimum of delay in processing.

Injection of crude toxin apparently produces a general paralysis. It appears to affect the nervous system, especially respiratory centers, before the muscular system. In the frog, the central nervous system is apparently affected before the peripheral nervous system. Crude toxin seems to alter the permeability of capillary walls in mice, fish and frogs. Hemolysis was not observed.

Since the toxicity of the capsule contents of *Physalia* is reduced by some organic solvents, and since these solvents also inactivate adherent nematocysts, the local application of alcohol to the skin of a swimmer stung by *Physalia* is an effective palliative measure.

Summary and Conclusions

The general composition and conditions of reactivity of the nematocysts and nematocyst contents of *Physalia* are described. A method is presented for isolation of nematocysts without contamination by other tentacular material. The nematocyst content appears to be a highly labile protein complex. The toxicity of the capsule contents is destroyed or denatured by heating to 60° C., by drying, by treatment with ethyl ether, acetone, or ethanol. Activity may be preserved for two months when the material is stored at − 5° C. The approximate lethal dose for mice, when the toxin was injected intraperitoneally, was 0.037 ml./kilo. of a preparation which contained 0.201% total N. The toxin was shown to be devoid of hemolytic activity for fish erythrocytes. When tested in fish, frogs or mice it appeared to affect the nervous system, particularly the respiratory centers, before voluntary muscles. Localized changes in cardiovascular tone have been observed in some test animals. *Physalia* toxin elicited responses in the isolated heart of the clam which were similar to those caused by acetylcholine.

LITERATURE CITED

Deichmann, Wm. B., and T. J. LeBlanc, 1943. Determination of the approximate lethal dose with about six animals. *J. Indust. Hyg.*, **25**: 415–417.

Glaser, O. C., and C. M. Sparrow, 1909. The physiology of nematocysts. *J. Exp. Zool.*, **6**: 361–382.

Lenhoff, H. M., E. S. Kline and R. Hurley, 1957. A hydroxyproline-rich, intracellular, collagen-like protein of *Hydra* nematocysts. *Biochim. Biophys. Acta*, **26**: 204–205.

Phillips, John H., 1956. Isolation of active nematocysts of *Metridium senile* and their chemical composition. *Nature*, **178**: 932.

Richet, Charles, and D. Portier, 1936. Recherches sur la toxine des coelenteres et les phénomènes d'anaphylaxie. *Result. Camp. Sci. Monac.*, **95**: 3–24.

Welsh, John H., 1956. *In:* Papers in Marine Biology and Oceanography. Pergamon Press Ltd., London. Pp. 287–297.

Welsh, John H., and R. Taub, 1948. The action of choline and related compounds on the heart of *Venus mercenaria*. *Biol. Bull.*, **95**: 346–350.